Low predictive value of intrinsic fibroblast radiosensitivity for fibrosis development following radiotherapy for breast cancer

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(Received 29 October 1997; accepted 10 February 1998)

Abstract.
Purpose: To test whether the intrinsic radiosensitivity of skin fibroblasts from breast cancer patients correlates with the degree of breast fibrosis after breast conserving therapy.
Methods: In a systematic study design, 79 patients were selected from an earlier study group of 385 patients based on observed fibrosis and seven identified clinical risk factors for fibrosis development. *In vitro* radiosensitivity of patients' dermal fibroblasts was determined by clonogenic assay of early passage cultures. Survival was determined after irradiation at 0, 2 and 4 Gy, given in two fractions of 2 Gy with a 6 h interval.
Results: There was a significant inter-patient variation for SF2 values (coefficient of variation = 40%). The ratio of SF2 values for fibroblasts from patients with breast fibrosis versus those without was 0·80 (95% CI: 0·60–1·07). This was a statistically non-significant trend ($p = 0{\cdot}13$). The same ratio for a derived value for SF2 ((SF2 + $\sqrt{SF4}$)/2) was 0·88 ($p = 0{\cdot}19$).
Conclusions: A significant variation in intrinsic radiosensitivity of breast cancer patients' dermal fibroblasts was observed. However, the degree of radiosensitivity did not show a significant correlation with fibrosis development. This indicates that the use of fibroblast radiosensitivity will have a limited usefulness for predicting fibrosis following breast irradiation.

1. Introduction

To be able to improve the therapeutic ratio of radiation treatment, it would be desirable to predict not only tumour response to radiation (West *et al.* 1991, Begg *et al.* 1992, Höckel *et al.* 1996), but also the severity of normal tissue reactions. In particular, a more accurate estimate of the risk of developing a given level of normal tissue reaction to radiotherapy for individual patients would be desirable. Several authors have discussed the rationale for such an approach (Norman *et al.* 1988, Ågren *et al.* 1990, West and Hendry 1992, Raaphorst 1993, Burnet *et al.* 1996, Peters 1996, Tucker *et al.* 1996, Bentzen 1997). For example, for a given treatment regimen a limited risk of a severe late reaction developing in the population of treated patients is accepted. An accurate prediction of which patients will form the subgroup that develops these late effects would mean that the estimation of the risk for these individuals would then be 100%. Such patients could be offered an alternative treatment option, e.g. lower dose, hyperfractionation, or another therapeutic modality. For the remaining patients, the dose could be increased to a level that is isoeffective for normal tissue complication risk and thus improve the therapeutic ratio for the patient group as a whole. This is most important if the tumour control probability is relatively low or moderate, i.e. the tumour control probability is near the bottom of a sigmoid dose–response curve.

Such considerations encourage the identification of sensitive and specific predictive factors for normal tissue reactions. Several groups have reported a promising association between late radiation reactions and an *in vitro* assay for fibroblast radiosensitivity (Geara *et al.* 1993, Burnet *et al.* 1994, Johansen *et al.* 1994, 1996, Brock *et al.* 1995). These studies have reported on a maximum of 32 patients and only one study (Johansen *et al.* 1996) included a correction for other factors influencing outcome. In 1994, Borger *et al.* 1994b developed a model to predict fibrosis based on clinical and treatment factors. This model does not perfectly predict fibrosis, which suggests other factors are also important. One hypothesis is that biological parameters such as patients' intrinsic radiosensitivity determine the radiation reaction of normal tissues. The *in vitro* radiosensitivity was therefore tested of fibroblasts obtained from 79 breast cancer patients following breast conserving therapy for whom the level of fibrosis had been determined together with a quantitative analysis of other identified risk factors for fibrosis development.

2. Patients, materials and methods

2.1. *Normal tissue end point and patient selection*

Borger *et al.* (1994b) reported on a retrospective study of 404 patients in which a semi-quantitative assessment was performed of fibrosis developing fol-

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0955–3002/98 $12.00

lowing breast conserving treatment carried out at the Netherlands Cancer Institute. Treatment consisted of tumour excision, axillary lymph node dissection and irradiation to the whole breast followed by a boost dose to the area of tumour excision with interstitial iridium. The external beam irradiation to the whole breast was given in daily fractions of 2 Gy to a total dose of 50 Gy with either cobalt-60 γ-rays or 4 or 8 MV X-rays. The boost with an iridium implant was given to a dose of 15–25 Gy (Borger *et al.* 1994a). For each patient the degree of fibrosis developing at the iridium boost site was assessed by palpation by a panel of four clinical observers, who compared the degree of induration in the treated breast with the same site on the contralateral untreated breast. The degree of fibrosis was assigned to one of four relative categories: no fibrosis (no difference between the two breasts); and grades 1, 2 and 3 fibrosis (a small, moderate and large difference, respectively). The inter-observer variation did not exceed more than one division on the scale. In case of inconclusive results, average scores were rounded up to the nearest integer. The median follow up of the study population at the time of the fibrosis assessment was 70 months (range 30–133 months).

Risk factors for fibrosis were identified using the proportional odds model in a polychotomous logistic regression. The following variables were included in the analysis: treatment period; age; follow-up period; fat : gland ratio on the mammogram; mammographic tumour size; clinical tumour size; tumour localization; specimen volume; breast infection; whole breast beam energy; implant dose; implant dose rate; normalized total dose, NTD_2 (see table 1); implant volume 100% (of the reference dose); implant volume 200%; number of sources; source spacing; and chemotherapy (adjuvant CMF). Seven independent patient and treatment related factors were identified that could be combined to give a 'predictive score' model for fibrosis development (table 1). However, the weighted kappa coefficient of agreement (Cicchetti and Allison 1971) between the predicted score and the observed fibrosis was still only 0·40, implying that factors other than those parameters studied to date could influence fibrosis. For this study of fibroblast radiosensitivity, two groups of 'residuals' were selected from the analysis of Borger *et al.* (1994b). The study design is set out in figure 1.

Table 1. Independent predictive factors for increased fibrosis development.

Older age
Longer follow-up duration
Higher normalized total dose (NTD_2)[a]
Larger clinical tumour size
Larger boost volume (100% reference dose)
Administration of adjuvant chemotherapy
Beam quality (Co-60 worse than 4–8 MV)

[a]NTD_2, the total dose given in fractions of 2 Gy which is biologically equivalent to the actual dose given according to the linear–quadratic model, using an α/β value of 2 Gy and 1·5 h for the recovery half life of sublethal damage repair. From Borger *et al.* 1994b.

The first study group comprised those patients who had developed a high or moderate level of fibrosis (observed categories 3 and 4), despite a relatively low 'predictive score' (predicted categories 1 and 2). This group was compared to a control group who had the same low predictive score but with no fibrosis development (observed category 1), as predicted by the model. The second study group were patients with a minimal level of fibrosis (observed categories 1 and 2), despite a high predictive score (predicted categories 3 and 4); they were compared with a control group with the same high-risk factors but the predicted severe level of fibrosis (observed categories 3 and 4).

From a total of 385 patients for whom all risk factors were known, 153 were identified as being in the study or control groups (figure 1). Thirty-two patients had in the intervening period between the two studies (5 years) either died, been lost to follow up or had become too ill. The remaining 121 patients were invited by letter to take part in the study of fibroblast radiosensitivity, and 82 agreed to donate a skin punch biopsy for fibroblast culture. The patients from whom no biopsy was obtained had a similar distribution over the study and control groups with and without fibrosis as those patients who took part

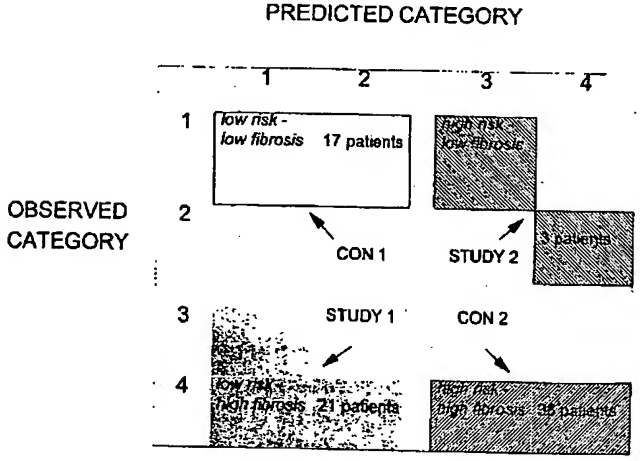

Figure 1. Selection of patient study and control groups from the whole patient group observed for the degree of fibrosis, based on the predicted level of fibrosis (level of risk factors for fibrosis) and the actual level of fibrosis observed.

in the study. The study protocol had been approved by the ethical and scientific committee of the Institute and all patients gave their written informed consent before the biopsy was taken.

2.2. *Cell culture techniques*

2.2.1. *Fibroblast outgrowth.* A punch skin biopsy for fibroblast culture was taken from the lateral side of the upper thigh, i.e. at a distance from the site of radiotherapy treatment. The skin was cleaned with 70% alcohol and the area was numbed with chlorethylene spray. The biopsy was placed in sterile phosphate-buffered saline (PBS) containing antibiotics for transfer to the laboratory. For culture, each skin biopsy was first treated for 20 s with 70% alcohol and washed in PBS, then cut into about six pieces and divided between two 5 cm Petri dishes. Carefully added to this, so that the pieces remained attached to the bottom, was 1·5 ml of medium containing serum. The dishes were then incubated at 37°C with 5% CO_2. The medium was replaced once a week, increasing the volume to 2 ml when outgrowth was evident after 1–2 weeks. Once extensive fibroblast outgrowth had been obtained after 2–3 weeks (passage 0), the skin pieces were removed and the cells trypsinized (0·05% trypsin) and passaged into a 25 cm^2 culture flask (Falcon) with 5 ml medium (passage 1). A second expansion passage in two 75 cm^2 flasks provided sufficient numbers of cells (about 5×10^6) for freezing in liquid nitrogen for later assays.

2.2.2. *Medium specifications.* For fibroblast outgrowth and the clonogenic assays Dulbecco's modified Eagle's medium (DMEM) was supplemented with 10% human serum, 100 μg/ml penicillin and 100 μg/ml streptomycin, as described previously (Begg *et al.* 1993).

2.3. *Radiation specifications and dosimetry*

Cells were irradiated as confluent monolayers in the culture flasks at room temperature using γ-rays from two opposing ^{137}Cs sources at a dose-rate of 0·85–0·80 Gy/min. Routine quality control of the dosimetry performed near the end of the study gave lower values for the dose rate than had been assumed based on earlier measurements and source decay calculations. Corrections were therefore applied based on the most recent measurements. In addition, corrections were applied for source decay over the 2·5 year period of these experiments. SF2 and SF4 values (surviving fractions at 2 and 4 Gy) were corrected by applying the linear–quadratic model of cell survival to convert dose difference to survival difference. An α/β value of 17 Gy was assumed, based on the median value from an earlier series of single dose survival curves (Begg *et al.* 1993). Within the irradiation chamber there was a maximum dose inhomogeneity of $\pm 10\%$.

2.4. *Clonogenic survival*

This assay was performed using a feeder layer protocol as previously described (Russell *et al.* 1995). Human fibroblast feeder cells from the same fibroblast strain as that being tested were irradiated to a dose of 32 Gy, trypsinized, diluted to a concentration of 6×10^4/ml and 1 ml added to a 9 mm Petri dish containing 8 ml of medium. Because of the large number of assays to be performed, a three-point survival curve was chosen for determination, with dose points at 0, 2 and 4 Gy. On day 2, control cells were mock irradiated (0 Gy). Cells for the 2 Gy dose point were irradiated as a confluent monolayer in a 25 cm^2 flask and immediately washed, trypsinized and resuspended in medium at four different cell concentrations and plated by adding 1 ml of the relevant cell concentration to the respective culture dish. For the 4 Gy dose point, cells were irradiated with two fractions of 2 Gy with an inter-fraction interval of 6 h and then plated out at three different cell concentrations. The assay was carried out in quadruplicate for the 0 Gy and in duplicate for the 2 and 4 Gy dose levels. All cultures were incubated for 14 days without a medium change and the resulting colonies were fixed and stained with crystal violet. The number of colonies consisting of more than 50 cells were counted and the surviving fraction calculated as the ratio of plating efficiencies for irradiated and unirradiated cells. The clonogenic survival was determined at least twice, and in some cases four times for each cell strain, using cells from consecutive or parallel passages.

2.5. *Statistical methods*

Surviving fraction data were log transformed before analysis (see below). A mixed effect analysis of variance (ANOVA) was performed to separate inter-assay and inter-patient variation, also taking possible systematic differences between passages into account. Passage number and fibrotic risk score was used as a fixed effect but patient identity as a random effect within the fibrotic risk score. As repeat assays were performed at the same passage level in some cases, the inter-assay variation could be decomposed into within-passage variation and between-passage variation. This was accomplished by introducing the

interaction between patient identity (within the fibrotic risk score) and passage number as a random effect into the model. Variance components were estimated by the method of moments, based on the (expected) mean square errors. To analyse the association between fibrosis and adjuvant hormonal treatment with tamoxifen, a proportional odds polychotomous logistic regression was used with the original predictive score function as described in Borger *et al.* (1994b) as an interval variable and tamoxifen treatment as a factor. The relationship between SF2 and the derived value for SF2 (see §3.4) and fibrosis development was determined by the statistical technique of analysis of variance using log transformed SF2 and derived SF2 data. Proc GLM and proc logistic of SAS 6.12 for Windows 95 were used for the calculations.

3. Results

3.1. *Cell cultures*

From a total of 82 biopsies primary outgrowth was obtained in 76, and after repeat biopsies in a further three. For three patients no or insufficient outgrowth was obtained, even with a second biopsy. Cell survival data were therefore obtained for a total of 79 patients' strains. Using the feeder cell layer technique and 10% human serum for colony outgrowth, an average plating efficiency of 8% with a range from 0·16% to 30·5% was obtained.

3.2. *Cell survival determinations*

A summary of all the survival curves obtained is given in figure 2. For each cell strain the average curve from replicate assays is shown. For clarity, the survival curves are divided between two panels, the top panel shows the results from the strains from patients in the study and control group 1 (low predictive score), and the bottom panel shows the survival curves from fibroblasts obtained from patients in the study and control group 2 (high predictive score). This summary of the survival data demonstrates the spread of results between patients and the variation in the shape of the survival 'curves'. The surviving fraction values at 2 Gy (SF2) varied from 0·723 to 0·031 with a mean value of 0·248. The surviving fraction at 4 Gy (SF4) varied from 0·135 to 0·009 (mean 0·047). For further analyses the SF2 values were log transformed. The choice of transformation was based on residual analyses. Apart from two apparent outliers (one case and one control from study group 1), residuals from the most extensive model were reasonably normally distributed and

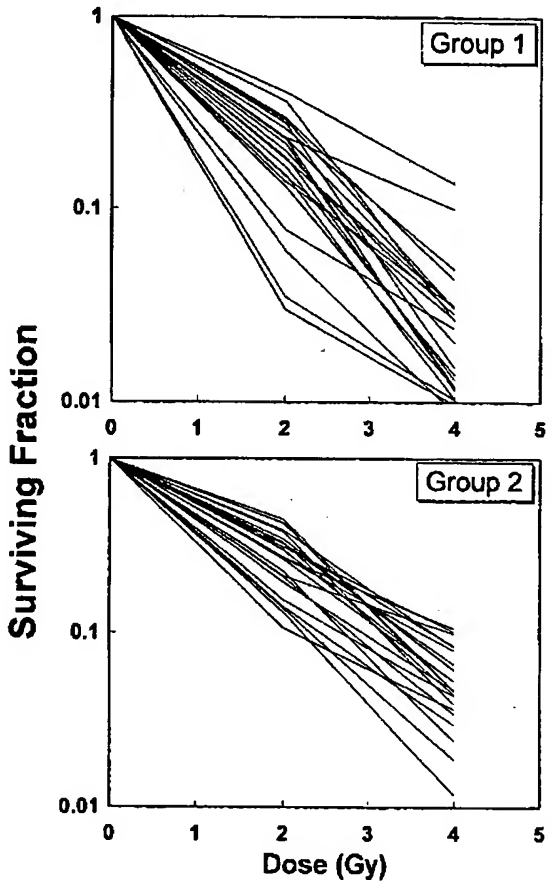

Figure 2. Summary of fibroblast cell survival curves, averaged per patient. Top panel: study and control patients from group 1 (low level of risk factors predictive for fibrosis). Bottom panel: patients from study and control group 2 (high level of risk factors for fibrosis).

the variances appeared to be stabilized after the transformation. Derived SF2 values, calculated from both crude SF2 and SF4 data, were also normally distributed after log transformation, and the log transformed values were used in the further analyses.

3.3. *Variation in radiosensitivity between and within patients' fibroblast strains*

Clonogenic survival was determined in two separate experiments for 61 cell strains, in three separate experiments for 14 cell strains, and four times for four strains. An example of repeated assays for an individual patient's strain is given in figure 3. The total coefficient of variation (CoV) for crude SF2 values, including inter-assay and inter-patient variation, was 52%. Strong evidence was found (p= 0·0001) that the observed variation between patients could not be attributed solely to the inter-assay variation (within patients). The estimated CoV for inter-assay variation was 39%, while the estimated

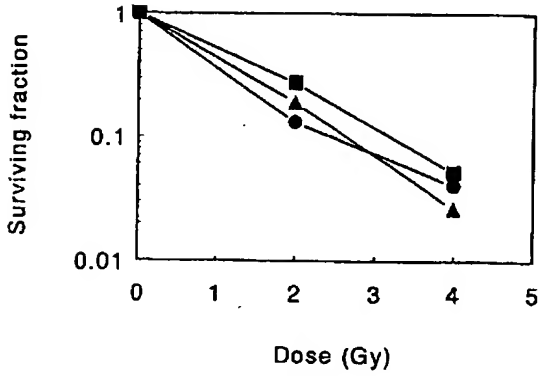

Figure 3. Example of three repeated cell survival determinations for a single fibroblast strain illustrating inter-assay variability.

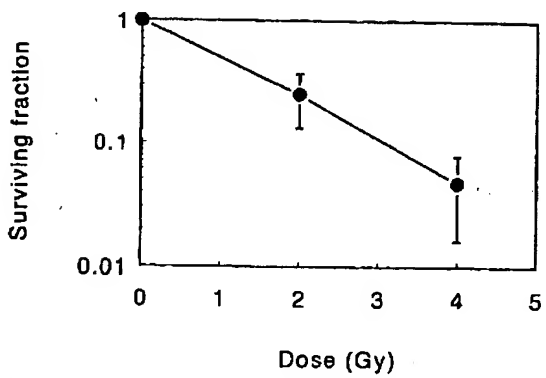

Figure 4. Mean cell survival at 2 and 4 Gy for assays on all strains, from both study and control patients. The mean for the whole patient group was derived from the mean values per patient. Error bars represent standard deviations; $n=178$ for the 2 Gy dose point and $n=175$ for the 4 Gy dose point.

net CoV for between-patient variation was 40% (i.e. after taking the inter-assay variation into account). The CoV for between-patient variation within a fibrotic score group was 34%. This analysis was based on 180 SF2 values for the inter-assay variation, 79 (averaged) values for the between-patient variation. There was no evidence for a systematic difference between passages ($p=0{\cdot}20$).

For SF4 values, however, no evidence was found ($p=0{\cdot}39$) that the observed variation between strains was mainly due to a true between-patient variation. The estimated CoV for inter-assay variation was 76%, while the estimated CoV for between-patient variation was 46% after correction for inter-assay variation, and 13% within a fibrotic score group. Again, no evidence was found for a systematic effect between passages, i.e. the variations were random and not systematic.

For the derived value of SF2 ($SF2+\sqrt{SF4}/2$ (see below), a between-patient variation of 30% was calculated, after taking an inter-assay variation of 39% into account. For this parameter there was also evidence that the observed variation could not be attributed solely to the inter-assay variation ($p=0{\cdot}0015$), i.e. there was a significant inter-patient variation for the derived value of SF2.

3.4. *Relationship between SF2 and SF4 estimations*

The dose of 4 Gy was given as two fractions of 2 Gy to confluent cell cultures. Assuming complete repair of sublethal damage in the 6 h inter-fraction interval and equal factors determining plating efficiency despite differences in cell density (Pomp *et al.* 1996), and no intra-assay variability, it would be expected that $SF2=\sqrt{SF4}$. The SF2 and SF2+2 give the first two points on a fractionated dose–survival curve. By combining both data points a figure is derived from what is in fact 3×2 Gy fractions, which it was thought would improve the accuracy of the estimated mean value of the survival at 2 Gy. An averaged (derived) value for SF2 could then be calculated as $(SF2+\sqrt{SF4})/2$. As can be deduced from inspection of the graphs in figure 2, the relationship between SF2 and SF4 varied between patients' strains; on the basis of the assumptions noted above a straight line would be expected for the survival 'curve'. The relationship between the survival at the two dose points is summed for 178 experiments in figure 4. This illustrates that the curve is approximately linear for the patient group as a whole, consistent with the hypothesis of nearly equal effects per fraction and complete repair. This is supported by the average ratio of SF2: $\sqrt{SF4}$ of 0·93 (CoV: 35%; $n=175$), on the basis of the log transformed values. The fact that the ratio is less than unity could be explained by the fact that the cells were trypsinized and immediately plated after the single 2 Gy dose and the second of the 2+2 Gy doses, but not after the first 2 Gy fraction. After the first 2 Gy fraction the cells were held in confluence for 6 h, giving them time for potentially lethal damage repair (PLDR), and thus a survival advantage over cells which were immediately trypsinized.

3.5. *Relationship between fibroblast radiosensitivity and fibrosis*

3.5.1. *Inclusion of tamoxifen use as a factor in the analysis.* There have been reports that tamoxifen treatment was associated with radiotherapy-induced lung and, possibly, subcutaneous fibrosis (Bentzen *et al.* 1996). Before analysing the relationship between fibrosis level and SF2, adjuvant hormonal treatment was therefore first included with tamoxifen

in the analysis of possible factors which could affect fibrosis development. No clear relationship between tamoxifen use and fibrosis was found (log odds ratio: 1·03; SE: 0·68; $p=0·14$). In view of the small number of patients with adjuvant hormonal treatment (11 patients) the power of this test was low. However, because of the relatively large observed odds ratio, subsequent analyses were adjusted for tamoxifen use.

3.5.2. *SF2 versus fibrosis.* The mean SF2 values for each study and control group are summarized in table 2 and the data for individual patients are given graphically in figure 5, which shows the mean SF2 values per patient. The upper panels in figure 5 show the cumulative frequency for SF2 values split between study and control groups for group 1 (low predictive score) and the lower panels for group 2 (high predictive score). This illustrates, in particular, the trend for patients with a low predictive score and greater than expected fibrosis to have low fibroblast SF2 values. Before analysing this apparent difference for statistical significance, it was first determined whether the relationship between SF2 and fibrosis depended on the predictive score (e.g. a low SF2 might predict for fibrosis only when the predictive score is low, or only when it is high; i.e. a predictive score — fibrosis interaction). From a model with the main factors being: (1) predictive score (1st–2nd quartile, low score, versus 3rd–4th quartile, high score); (2) fibrosis (no or small difference with untreated breast versus moderate to large difference); (3) tamoxifen treatment (yes or no); and (4) interaction between the predictive score and fibrosis, no evidence was found for the interaction ($p=0·37$). In other words, if there is a relation between SF2 and fibrosis, this does not seem to vary between the two categories of the predictive score. This meant that data from both study and control groups could be used together in the same analysis.

Considering all the data and eliminating the interaction from the model, only a statistically non-significant trend was found for a relationship between SF2 and fibrosis ($p=0·13$, adjusted for predictive score and hormonal treatment). The estimated ratio of SF2 in the fibrotic group to that of the non-fibrotic group, averaged over the two score categories, was 0·80 (95% CI: 0·60–1·07). This indicates that, on average, fibroblasts from patients showing more than expected fibrosis showed a trend to being more radiosensitive. However, SF2 values were lower in the first two quartiles of the predictive score ($p=$ 0·0001, adjusted for fibrosis and adjuvant tamoxifen). Further analysis showed that this relationship was mainly (but not completely) due to a low SF2 in patients given lower doses (NTD_2 <70 Gy, one of the main factors contributing to the low predictive score; $p=0·0017$, adjusted for observed fibrosis and hormonal treatment). After further adjustment for NTD_2, the relationship between a low SF2 and the predictive score was less statistically significant, $p=$ 0·027. No evidence of a relationship between SF2 and adjuvant tamoxifen was found.

Table 2. Mean values for surviving fraction at 2 Gy (SF2) for each study and control group. The numbers in brackets refer to the number of patients in the group.

	Predictive score quartiles.	
Observed fibrosis	1 and 2 (low risk)	3 and 4 (high risk)
No/minimal	mean: 0·228 range: 0·035–0·408 Control group 1 (17)	mean: 0·297 range: 0·199–0·423 Study group 2 (3)
Moderate/large	mean: 0·174 range: 0·031–0·300 Study group 1 (21)	mean: 0·295 range: 0·107–0·723 Control group 2 (38)

3.5.3. *Derived SF2 values versus fibrosis.* A similar analysis was performed on the log transformed values for derived SF2, $(SF2 + \sqrt{SF4})/2$. Again, no evidence was found for an interaction for derived SF2 values between predictive score and fibrosis. Considering all the data and eliminating the interaction from the model, no evidence was found for a relationship between derived SF2 and fibrosis ($p=0·19$, adjusted for predictive score and hormonal treatment). The estimated ratio of derived SF2 in the fibrotic group versus the non-fibrotic group was 0·88 (95% CI: 0·72–1·07). This shows the same statistically insignificant trend as for the crude SF2 values, although the p-value is greater. As with the crude SF2 values, the derived SF2 was lower in the first two quartiles of the predictive score ($p<0·0001$, adjusted for fibrosis and tamoxifen use). This was due mainly to patients who had been treated to an NTD_2 of less than 70 Gy ($p=0·0002$, after adjustment for observed fibrosis and hormonal treatment). No evidence of a relationship was found between derived SF2 and tamoxifen in any of the analyses. The discrimination between clinically radiosensitive and radioresistent patients is therefore poorer with the derived SF2 data than with the crude SF2 data, and this is illustrated in figure 5, bottom panels, which shows very little difference between the cumulative frequency curves for the study and control groups.

A separate analysis was not conducted of the relationship between the SF4 values and fibrosis in order to avoid multiple statistical tests which could increase the possibility of deriving a statistically signi-

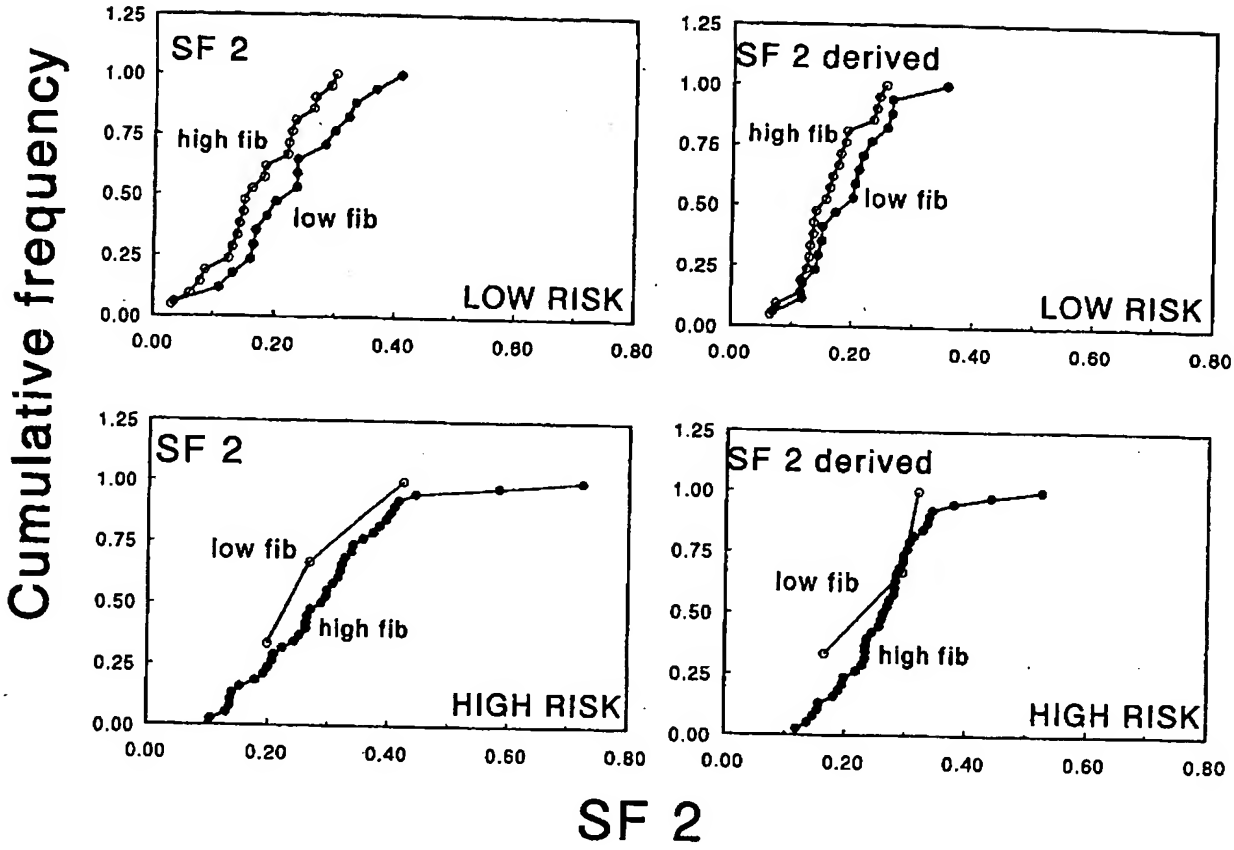

Figure 5. Cumulative frequency of crude SF2 values (left-hand panels) and derived SF2 values (right-hand panels), for study and control patients respectively. Top panels: patients from group 1 (low risk). Bottom panels: patients from group 2 (high risk). This illustrates the trend to lower SF2 values in patients with breast fibrosis for group 1 in particular.

ficant result 'by chance'. However, as the 'derived' value for SF2 shows an even poorer correlation with fibrosis than the 'crude' values, it seems unlikely that an analysis of SF4 values versus fibrosis would change the conclusions of the study.

4. Discussion

4.1. *Fibroblast radiosensitivity versus late normal tissue damage*

The findings of the present study, which reveal a non-significant trend for a low SF2 for fibroblasts from patients with greater than expected fibrosis, are compatible with the hypothesis that fibroblast SF2 correlates with observed breast fibrosis, because of the observed confidence interval. The authors' data are therefore also consistent with the data from earlier studies reported by Burnet *et al.* (1992, 1994), Brock *et al.* (1995), Geara *et al.* (1993) and Johansen *et al.* (1994, 1996), who reported associations between a low value for an *in vitro* survival parameter and the severity of a late normal tissue reaction (in most cases telangiectasia or fibrosis).

Patient and treatment related factors such as age, follow-up duration and dose intensity are now well documented as factors which influence radiation-induced normal tissue reactions, particularly skin reactions and fibrosis (Bentzen *et al.* 1990, Turesson 1990, Turesson *et al.* 1996). Any independent influence of intrinsic fibroblast radiosensitivity can only be adequately assessed after correction of the clinical parameter, in this case fibrosis, for known confounding factors. The present study investigates a patient population that had been extensively analysed to identify clinical factors influencing fibrosis development and selected two groups of patients that were identified as 'residuals' from the analysis, i.e. those patients who developed a greater or lesser degree of fibrosis than expected from the model. The fibroblast radiosensitivity was then compared to that of control groups who had the same level of risk factors for fibrosis development but an observed level of fibrosis that conformed with the predictive score. The relatively large number of patients included in this study, and the fact that seven other risk factors for fibrosis were taken into account in the study design, makes

the conclusions more applicable to a general radiotherapy patient population (in this case breast cancer patients), than studies based on ranking statistics. At the Institute of Cancer Research, Sutton, UK, a broadly similar study of fibroblast radiosensitivity from a case–control study of patients who have undergone breast conserving therapy is being carried out. A combined analysis of the two studies would have a greater statistical power. However, at the time of writing the results from the UK study are not yet known (J. Peacock, personal communication).

A direct comparison of the present study with the other reports mentioned above is made somewhat difficult since this study used the clinical endpoint of breast fibrosis as determined by palpation rather than endpoints such as telangiectasia (Burnet *et al.* 1994, Brock *et al.* 1995), subcutaneous fibrosis after mastectomy (Johansen *et al.* 1996) or a mixture of various epithelial and mesenchymal tissues (Geara *et al.* 1993). Bentzen *et al.* (1993) have addressed the lack of correlation between two late end points (fibrosis and telangiectasia) in the same patient, so possibly an *in vitro* parameter should not be expected to correlate with both clinical end points. It is possible that clinically observed fibrosis in breast tissue has different factors influencing its pathogenesis than in the skin.

Because of the large confidence interval for the ratio of SF2 values for patients with or without fibrosis development, the data are also compatible with the null hypothesis that there is no real statistical difference in fibroblast SF2 values between patients who do or do not develop radiation-induced breast fibrosis. Brock *et al.* (1995) also concluded from their comparison of fibroblast SF2 and acute and late skin reactions (telangiectasia) that there was 'no unambiguous evidence' for a correlation between the two parameters. There was a trend for an inverse SF2–telangiectasia correlation, but this was based on very small patient numbers.

In summary, the findings of the present study do not yet support the use of fibroblast radiosensitivity testing for predicting breast fibrosis in clinical practice. Reports that have attempted to quantify the benefits that could be obtained in terms of dose modification to improve the therapeutic ratio of radiation treatment (Tucker *et al.* 1996, Bentzen 1997) have assumed an assay accuracy and discriminatory power for SF2 (or other *in vitro* parameter) which is far greater than is possible in practice. It remains unclear whether stratifying patients further for other possible risk factors, i.e. reducing the 'noise' in the clinical data, or increasing the *in vitro* assay accuracy would increase its predictive power, or whether factors other than intrinsic fibroblast sensitivity would still dominate the response.

4.2. *Choice of normal tissue end point*

The end point of fibrosis at the site of the interstitial boost dose with iridium was a choice based on a number of considerations. First, since the *in vitro* radiation sensitivity of patients' fibroblasts was being tested, it was appropriate to use a tissue response that is probably related to fibroblast function. Other end points used in assessing the normal tissue (cosmetic) results of breast conserving therapy, such as measurements of nipple retraction, include a component of fibrosis, but also factors such as the site and orientation of the surgical scar. A second consideration was that detailed knowledge of the dose received by the breast tissue at the implant site was available. As the dose–response relationship for normal tissue effects in individuals is quite steep (Turesson 1990), and as the dose received is the most important determinant of fibrosis development (Borger *et al.* 1994b), it was relevant to assess the end point at the site where the dose was accurately known. Thirdly, the dose–response relationship for fibrosis for the patient population studied is shallow at 50 Gy and becomes steeper at dose levels of around 65–75 Gy, so there was a better distribution of the end point of fibrosis which could be observed.

4.3. *Comparison of* in vitro *data*

For the *in vitro* radiation survival curves three dose points of 0, 2 and 4 Gy were used. To obtain a better range of results for use in the analysis with the clinical data the 4 Gy dose was fractionated into two fractions of 2 Gy. A fractionated 4 Gy dose point was used as an alternative to a low-dose radiation, which has been reported by some groups to increase the spread of fibroblast radiosensitivity between cell strains, presumably by exploiting differences in repair capability, when compared to a high-dose rate (Nagasawa *et al.* 1992, Burnet *et al.* 1994, 1996). This finding is not universal however, since other reports have not confirmed that the use of a low-dose rate helped to provide a better distinction between cell strains of differing radiosensitivities (Little and Nove 1990, Geara *et al.* 1992, Brock *et al.* 1995). In the present study a greater survival was observed, on average, per 2 Gy at the fractionated 4 Gy dose point compared with the single 2 Gy dose, suggesting the effect of a repair phenomenon (PLDR), and also an increase in the range of results, reflected in the greater coefficient of variation (CoV) for the 4 Gy data (46%) compared with the 2 Gy dose point (40%). The greater CoV at 4 Gy could also be due to the total dose since the figures from this study compare with those obtained by Johansen *et al.* (1996) who

established survival curves with graded single doses and found a CoV for 2 Gy of 31% and for 3.5 Gy of 57%, although the exact figures cannot be directly compared because in this study 4 Gy was not given as a single dose and also due to differences in calculation techniques. Because the daily fraction dose for the external beam treatment had been 2 Gy, an average value was derived for the survival at 2 Gy from $(SF2 + \sqrt{SF4})/2$. The relationship between SF2 and SF4 which was not always consistent between patient strains could be due to variation in repair capacity, plating efficiency effects at different cell concentrations and assay variability. This change in the rank order between different patients at two dose levels was also noted by Johansen *et al.* (1994), and may partly explain why the derived value of SF2 had a lower CoV (30%) and did not show a better correlation with patients' fibrosis development than the crude SF2 values.

A significant inter-patient variation in SF2 and derived SF2 was observed, after inter-assay variation was taken into account. This supports the findings of other authors (Geara *et al.* 1992, Johansen *et al.* 1996), who calculated lower absolute values for inter-assay variation than was found in the present study; this can be attributed partly to the use of the crude survival data points from individual assays in this study's analyses, instead of SF2 values calculated from curve fits. The observation that the fibroblast strains with a low predictive score tended to have the lowest SF2 and SF4 values is not fully explained. A batch effect could be part of the explanation; the fibroblasts from patients in group 1 (low predictive score) were assayed first, followed by the fibroblasts from patients in group 2. Factors such as variations in serum or medium quality could play a role. It is not due to patients being given a lower dose if they developed a severe acute reaction; the difference in total dose between patients was almost entirely due to the dose of the boost given with an iridium implant about 3 weeks after the end of the external beam treatment to the whole breast. The dose given by the implant was determined before the start of radiotherapy. Neither was it related to other patient related factors that determined the predictive score, such as patient age. The observation of lower SF2 values for group 1 as a whole does not alter the conclusions that can be drawn from the study because of the way in which the study was designed and analysed.

4. Conclusion

Patients' intrinsic radiosensitivity, as determined by colony forming assays of dermal fibroblasts, did not show a significant correlation with the observed level of fibrosis development in the breast. Other patient and treatment related factors have greater predictive power. Possibly other *in vitro* tests could provide better discrimination between patients who develop greater or lesser degrees of fibrosis. Certainly (radio)biological factors other than pure radiosensitivity of fibroblasts need to be considered. The discrepancy between the α/β value for clinically observed fibrosis (low) and the average α/β value for fibroblasts derived from *in vitro* cell survival curves (high) implies that biological mechanisms other than fibroblast cell kill alone modify the radiation response seen *in vivo*. Currently the authors are testing whether an assay of fibroblast differentiation induction following irradiation correlates better with *in vivo* fibrosis development.

References

Ågren, A., Brahme, A. and Turesson, I., 1990, Optimization of uncomplicated control for head and neck tumors. *International Journal of Radiation Oncology, Biology, Physics*, **19**, 1077–1085.

Begg, A. C., Hofland, I., Van Glabbeke, M., Bartelink, H. and Horiot, J. C., 1992, Predictive value of potential doubling time for radiotherapy of head and neck tumour patients: results from the EORTC cooperative trial 22851. *Seminars in Radiation Oncology*, **1**, 22–25.

Begg, A. C., Russell, N. S., Knaken, H. and Lebesque, J. V., 1993, Lack of correlation of human fibroblast radiosensitivity *in vitro* with early skin reactions in patients undergoing radiotherapy. *International Journal of Radiation Biology*, **64**, 393–405.

Bentzen, S. M., Turesson, I. and Thames, H. D., 1990, Fractionation sensitivity and latency of telangiectasia after postmastectomy radiotherapy: a graded response analysis. *Radiotherapy and Oncology*, **18**, 95–106.

Bentzen, S. M., Overgaard, J. and Overgaard, M., 1993, Clinical correlations between late normal tissue endpoints after radiotherapy: implications for predictive assays of radiosensitivity. *European Journal of Cancer*, **29A**, 1373–1376.

Bentzen, S. M., Skoczylas, J. Z., Overgaard, M. and Overgaard, J., 1996, Radiotherapy-related lung fibrosis enhanced by tamoxifen. *Journal of the National Cancer Institute*, **88**, 918–922.

Bentzen, S. M., 1997, Potential clinical impact of normal-tissue intrinsic radiosensitivity testing. *Radiotherapy and Oncology*, **43**, 121–131.

Borger, J., Kemperman, H., Hart, A., Peterse, J., Van Dongen, J. and Bartelink, H., 1994a, Risk factors in breast conservation therapy. *Journal of Clinical Oncology*, **12**, 653–660.

Borger, J. H., Kemperman, H., Sillevis Smitt, H., Hart, A., Van Dongen, J., Lebesque, J. and Bartelink, H., 1994b, Dose and volume effects on fibrosis after breast conservation therapy. *International Journal of Radiation Oncology, Biology, Physics*, **30**, 1073–1081.

Brock, W. A., Tucker, S. L., Geara, F. B., Turesson, I., Wike, J., Nyman, J. and Peters, L. J., 1995, Fibroblast radiosensitivity versus acute and late normal skin

responses in patients treated for breast cancer. *International Journal of Radiation Oncology, Biology, Physics*, **32**, 1371–1379.

BURNET, N. G., NYMAN, J., TURESSON, I., WURM, R., YARNOLD, J. R. and PEACOCK, J. H., 1992, Prediction of normal-tissue tolerance to radiotherapy from *in vitro* cellular radiation sensitivity. *The Lancet*, **339**, 1570–1571.

BURNET, N. G., NYMAN, J., TURESSON, I., WURM, R., YARNOLD, J. R. and PEACOCK, J. H., 1994, The relationship between cellular radiation sensitivity and tissue response may provide the basis for individualising radiotherapy schedules. *Radiotherapy and Oncology*, **33**, 228–238.

BURNET, N. G., WURM, R., NYMAN, J. and PEACOCK, J. H., 1996, Normal tissue radiosensitivity — How important is it? *Clinical Oncology*, **8**, 25–34.

CICCHETTI, D. V. and ALLISON, T., 1971, A new procedure for assessing reliability of scoring EEG sleep recordings. *American Journal of EEG Technology*, **11**, 101–109.

GEARA, F. B., PETERS, L. J., KIAN ANG, K., WIKE, J. L., SIVON, S. S., GUTTENBERGER, R., CALLENDER, D. L., MALAISE, E. P. and BROCK, W. A., 1992, Intrinsic radiosensitivity of normal human fibroblasts and lymphocytes after high- and low-dose-rate irradiation. *Cancer Research*, **52**, 6348–6352.

GEARA, F. B., PETERS, L. J., KIAN ANG, K., WIKE, J. L. and BROCK, W. A., 1993, Prospective comparison of *in vitro* normal cell radiosensitivity and normal tissue reactions in radiotherapy patients. *International Journal of Radiation Oncology, Biology, Physics*, **27**, 1173–1179.

HÖCKEL, M., SCHLENGER, K., ARAL, B., MITZE, M., SCHAFFER, U. and VAUPEL, P., 1996, Association between tumour hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Research*, **56**, 4509–4515.

JOHANSEN, J., BENTZEN, S. M., OVERGAARD, J. and OVERGAARD, M., 1994, Evidence for a positive correlation between *in vitro* radiosensitivity of normal human skin fibroblasts and the occurrence of subcutaneous fibrosis after radiotherapy. *International Journal of Radiation Biology*, **66**, 407–412.

JOHANSEN, J., BENTZEN, S. M., OVERGAARD, J. and OVERGAARD, M., 1996, Relationship between the *in vitro* radiosensitivity of skin fibroblasts and the expression of subcutaneous fibrosis, telangiectasia, and skin erythema after radiotherapy. *Radiotherapy and Oncology*, **40**, 101–109.

LITTLE, J. B. and NOVE, J., 1990, Sensitivity of human diploid fibroblast cell strains from various genetic disorders to acute and protracted radiation exposure. *Radiation Research*, **123**, 87–92.

NAGASAWA, H., LITTLE, J. B., TSANG, N. M., SAUNDERS, E., TESMER, J. and STRNISTE, G. F., 1992, Effect of dose rate on the survival of irradiated human skin fibroblasts. *Radiation Research*, **132**, 375–379.

NORMAN, A., KAGAN, A. R. and CHAN, S. L., 1988, The importance of genetics for the optimization of radiation therapy. *American Journal of Clinical Oncology*, **11**, 84–88.

PETERS, L. J., 1996, Radiation therapy tolerance limits for one or for all? *Cancer*, **77**, 2379–2385.

POMP, J., WIKE, J. L., OUWERKERK, I. J. M., HOOGSTRATEN, C., DAVELAAR, J., SCHRIER, P. I., LEER, J. W. H., THAMES, H. D. and BROCK, W. A., 1996, Cell density dependent plating efficiency affects outcome and interpretation of colony forming assays. *Radiotherapy and Oncology*, **40**, 121–125.

RAAPHORST, G. P., 1993, Prediction of radiotherapy response using SF2: is it methodology or mythology? *Radiotherapy and Oncology*, **28**, 187–188.

RUSSELL, N. S., ARLETT, C. F., BARTELINK, H. and BEGG, A. C., 1995, Use of fluorescence *in situ* hybridization to determine the relationship between chromosome aberrations and cell survival in eight human fibroblast strains. *International Journal of Radiation Biology*, **68**, 185–196.

TUCKER, S. L., GEARA, F. B., PETERS, L. J. and BROCK, W. A., 1996, How much could the radiotherapy dose be altered for individual patients based on a predictive assay of normal-tissue radiosensitivity? *Radiotherapy and Oncology*, **38**, 103–113.

TURESSON, I., 1990, Individual variation and dose dependency in the progression rate of skin telangiectasia. *International Journal of Radiation Oncology, Biology, Physics*, **19**, 1569–1574.

TURESSON, I., NYMAN, J., HOLMBERG, E. and ODEN, A., 1996, Prognostic factors for acute and late skin reactions in radiotherapy patients. *International Journal of Radiation Oncology, Biology, Physics*, **36**, 1065–1075.

WEST, C. M. L., DAVIDSON, S. E., HENDRY, J. H. and HUNTER, R. D., 1991, Prediction of cervical carcinoma response to radiotherapy. *The Lancet*, **338**, 818.

WEST, C. M. L. and HENDRY, J. H. L., 1992, Intrinsic radiosensitivity as a predictor of patient response to radiotherapy. *British Journal of Radiology*, Suppl. 24, 146–152.